Megakaryocyte Growth and Development Factor Stimulates Enhanced Platelet Recovery in Mice After Bone Marrow Transplantation

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Megakaryocyte growth and development factor (MGDF) is a recently characterized ligand for the cell surface receptor mpl. We have evaluated the effects of polyethylene glycolated recombinant human MGDF (PEG-rHuMGDF) on recovery of hematopoietic cells in mice following bone marrow transplantation (BMT) to support lethal irradiation. Mice treated with PEG-rHuMGDF (50 μg/kg/d) had accelerated recovery of platelet numbers compared with BMT mice treated with carrier or recombinant human granulocyte colony-stimulating factor (rHuG-CSF, 72 or 200 μg/kg/d). In contrast, PEG-HuMGDF had no effect on white blood cell (WBC) or red blood cell (RBC) recovery. As previously reported, animals treated with rHuG-CSF had an enhanced recovery of WBC but not platelet or RBC levels. Interestingly, BMT recipient mice treated with the combination of PEG-rHuMGDF and rHuG-CSF showed simultaneous enhanced recovery of both leukocytes and platelets. PEGylated rHuMGDF was found to be considerably more potent than non-PEGylated rHuMGDF in this setting. PEG-rHuMGDF is an effective growth factor for enhancing platelet recovery in mice following BMT either alone or in combination with rHuG-CSF. It will be of interest to evaluate in a clinical setting the ratios of PEG-rHuMGDF and rHuG-CSF for simultaneous administration of these factors and accelerated recovery of both leukocytes and platelets.

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FOLLOWING MYELOABLATIVE therapy or bone marrow transplant (BMT), a period of pancytopenia occurs that involves increased risk of infections, febrile episodes, and bleeding. It is now widely established that the administration of hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) after BMT can shorten the period of pancytopenia. This has been shown to significantly decrease the risk of infection and the need for antibiotic support.

Recently, several groups have cloned the ligand for the cytokine receptor Mpl, or thrombopoietin. Mpl ligand has been shown to influence megakaryocyte growth and platelet production in vitro and in vivo in mice and primates. This material clearly offers clinical potential in settings where platelet numbers are low; these initial studies were designed to determine whether a truncated form of the Mpl ligand, MGDF, administered post-BMT can improve the rate and/or degree of platelet recovery.

In the clinical setting of high dose chemotherapy and stem cell transplant, accelerated recovery of all blood cell populations is obviously desirable and G-CSF, particularly, has proven very useful in improving the recovery of neutrophils. Since megakaryocyte growth and development factor (MGDF) offers the potential to improve platelet recovery also, we were interested in addressing the possibility of combination growth factor therapy in assisting the simultaneous recovery of both neutrophils and platelets after BMT. To do this we have treated mice simultaneously with recombinant human G-CSF (rHuG-CSF) in combination with polyethylene glycol (PEG) rHuMGDF in the recovery phase after BMT.

MATERIALS AND METHODS

BMT

Eight- to 12-week-old female BDF1 mice (C57Bl/6 × DBA, F1) were irradiated at 1,200 cGy (137Cs, dose rate 106.7 cGy/min) administered as a split dose (2 × 600 cGy, 4 hours apart). Bone marrow cells were harvested from littermate mice by flushing the femoral contents with Hank's Balanced Salt Solution (HBSS; Life Technologies Inc, Grand Island, NY) supplemented with 2% fetal bovine serum (Camsera International Inc, Rexdale, Ontario, Canada). Cells were counted in a hemocytometer and dilutions made to allow the intravenous injection of 106 bone marrow cells in 500 μL into irradiated mice 4 hours after completion of the irradiation.

Growth Factors

rHuMGDF was expressed in Escherichia coli using a plasmid that encodes a truncated form of the Mpl ligand, including the erythropoietin-like amino terminus. The material was further modified by the addition of poly(ethylene glycol) (PEG). rHuG-CSF (r-metHu-G-CSF, HuG-CSF) was prepared as previously described in E coli and purified to >99% purity. All growth factors were prepared at appropriate dilutions to give the dose levels indicated in the Results section. A dose of 72 or 200 μg/kg/d of rHuG-CSF was used alone or in combination with rHuMGDF at 150 μg/kg/d and PEG-rHuMGDF at 50 μg/kg/d. Treatment was administered as indicated in Results, by continuous subcutaneous infusion via Alzet mini-osmotic pump (2002, Alza Corp, Palo Alto, CA). Pumps were implanted the day before irradiation and BMT. Delivery from these pumps could be expected to begin within 4 hours and continue for up to 18 days. Carrier solution was phosphate buffered saline (PBS; Life Technologies) supplemented with 0.1% bovine serum albumin (BSA; Sigma, St Louis, MO) and was used to dilute growth factors for administration, or administered alone to carrier-treated animals.

Peripheral Blood Analysis

To determine recovery post-BMT, peripheral blood was collected from the retro-orbital sinus of mice at various days up to day 21. Full blood counts were performed on a Technicon H-1E (Technicon Instruments Corp, Tarrytown, NY) calibrated for mouse blood.

Statistical Analysis

Where indicated in the figures differences between carrier- and growth-factor-treated or between various treatment groups was tested with Student's t-test using the Sigma Stat program (Jandel Scientific, San Rafael, CA).
Platelet Recovery

rHuMGDF treatment. rHuMGDF administered to mice for 17 days after irradiation and BMT stimulated faster recovery of platelets than was seen in carrier treated animals (Fig 1). Continuous infusion offered an improvement of about 1.5-fold over the response obtained from the same dose administered by injection as measured by peak platelet numbers in normal mice.13 After irradiation and BMT (109 cells), the number of platelets in recipient mice fell over a 7-day period from a control value of 1,349 x 10^3 ± 113/µL (SD, n = 15) to <200 x 10^3/µL (Fig 1). Mice that received no rHuMGDF had a prolonged nadir (with counts <200) up to day 10. By day 12, recovery had started in mice that received marrow alone. A steady improvement in platelet numbers proceeded up to day 16, when numbers around 1,000 x 10^3/µL indicated a stable recovery phase, some 20% below pretreatment values, which was maintained beyond day 20. In comparable mice that received rHuMGDF support, recovery started sooner, 200 x 10^3/µL being exceeded on day 9 (compared with around day 11 in carrier treated animals, Fig 1). Platelet numbers then increased more quickly in rHuMGDF treated mice, and levels over 1,000 x 10^3/µL were noted by day 14, when platelet counts in control mice had reached around 700 x 10^3/µL. Normal (pretreatment) platelet numbers were seen at day 19 in mice that received 150 µg/kg/d non-PEGylated rHuMGDF given in parallel. The treatment of bone marrow recipients with PEG-rHuMGDF initiated platelet recovery on a shortened time scale similar to rHuMGDF treatment (Fig 1). By day 9, when control mice were still in the prolonged nadir at around 100 x 10^3 platelets/µL, PEG-rHuMGDF recipients had more than 300 x 10^3/µL. This advantage was retained for the duration of the study, with 1,000 x 10^3/µL being exceeded around day 12. Platelet numbers continued to increase up to 2,500 x 10^3/µL on day 16, at which point the osmotic pump began to fail and platelet numbers subsequently fell back toward normal.

Combination treatment. As previously reported rHuG-CSF did not accelerate platelet recovery in BMT recipients (Figs 2 and 3). One of our aims in these experiments was to ascertain the treatment schedules required for simultaneous stimulation of accelerated recovery of both neutrophils and platelets. To this end we attempted combination therapy with rHuG-CSF in addition to rHuMGDF or PEG-rHuMGDF. Although rHuMGDF had, as a single agent, improved platelet recovery above carrier treatment levels, little improvement over the control platelet response was seen in mice receiving rHuG-CSF + rHuMGDF (Figs 1 and 2). As stated above, PEG-rHuMGDF was the more potent of the two MGDF forms studied and the combination of PEG-rHuMGDF and rHuG-CSF was the most effective combination treatment in improving recovery of platelets in BMT recipients (Figs 2 and 3). Platelet counts below 200 x 10^3/µL were seen on only a single study day (day 7). At all other times, platelet counts in this group were higher than in other groups, indicating a reduced duration of thrombocytopenia, and markedly accelerated platelet recovery. Data
Fig 2. Mice received innocuous carrier, rHuG-CSF at 72 µg/kg/d, rHuMGDF at 150 µg/kg/d, PEG-rHuMGDF at 50 µg/kg/d, or combinations of these. Fifteen individual mice were assessed at each time point. Data are combined from three independent experiments (5 recipient mice per experiment per point) ± 1 SE. Platelet count in normal, nonirradiated, nontransplanted mice was $1,349 \times 10^3 \pm 113/\mu L$; WBC count was $10.65 \pm 2.95 \times 10^3/\mu L$. Points that differ significantly from carrier are denoted thus: *P < .05, **P < .01.

Illustrated in Fig 4 show that recipients of rHuG-CSF/PEG-rHuMGDF combination treatment had platelet recovery rates that were marginally slower than the rates observed in mice treated with only PEG-rHuMGDF. This slowing only became significant at a limited number of study days. The time to any specific level (eg, 200, 500, or $1,000 \times 10^3$ platelets/µL) and final platelet counts attained were not significantly different.

Fig 3. Mice received innocuous carrier, rHuG-CSF at 200 µg/kg/d, rHuMGDF at 150 µg/kg/d, PEG-rHuMGDF at 50 µg/kg/d, or combinations of these. Fifteen individual mice were assessed at each time point. Data are combined from three independent experiments (5 recipient mice per experiment per point) ± 1 SE. Platelet count in normal, nonirradiated, nontransplanted mice was $1,349 \times 10^3 \pm 113/\mu L$; WBC count was $10.65 \pm 2.95 \times 10^3/\mu L$. Points that differ significantly from carrier are denoted thus: *P < .05, **P < .01.

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WBC Recovery

In normal mice, in keeping with previously published data,11,12,16,17 neither rHuMGDF nor PEG-rHuMGDF affected leukocyte numbers, even at relatively high doses. Our experience with bone marrow graft recipients and rHuMGDF indicates that little effect on leukocyte populations is seen in this situation also (Fig 1). However, the doses infused have not exceeded 150 μg/kg/d rHuMGDF or 50 μg/kg/d PEG-rHuMGDF, and so we are unable to state explicitly whether any increase in leukocytes might be expected with either MGDF at higher doses.

rHuG-CSF accelerates neutrophil recovery in BMT recipients. Carrier, rHuMGDF, and PEG-rHuMGDF treated mice showed identical leukocyte recovery, both in terms of recovery rate and maximum circulating WBC count attained (Fig 2). The infusion of rHuG-CSF at either 72 or 200 μg/kg/d stimulated accelerated recovery of WBC. Leukocyte numbers below 1 x 10^3/μL, indicative of serious leukopenia, were exceeded around day 8 in recipients supported with rHuG-CSF. Accelerated recovery was then noted up to day 14 to 16, when treatment was stopped. The degree of leukocytosis induced by rHuG-CSF was equivalent at 72 or 200 μg/kg/d treatment levels. Despite the similar effects on WBC, platelet numbers were more markedly reduced by the higher dose, with the lower dose having no significant effect on platelet numbers (Figs 2 and 3).

DISCUSSION

Historically, platelets have proven a more difficult blood cell population in which to induce accelerated recovery after BMT, and patients may require the support of platelet transfusions for a number of days (eg, Kanz et al18). Several candidate growth factors have been tested in posttransplant and model systems with only limited success. In various settings interleukin-1 (IL-1),19 IL-3,20 IL-6,21 IL-11,22 and erythropoietin23,24 have all been suggested to increase circulating platelet counts, but the minimal efficacy or possible toxicity of these growth factors may limit their application.

The recent development of MGDF as a potential therapeutic for thrombocytopenia has raised the possibility of significantly improving the recovery of platelets in BMT recipients as well as in idiopathic thrombocytopenias. We administered rHuMGDF and a PEGylated derivative to mice after irradiation and BMT and found a significant improvement in the recovery of platelet numbers. The PEGylated material was more effective despite being administered at 50 μg/kg/d compared with 150 μg/kg/d for non-PEGylated material (Fig 1). Thus the PEGylated derivative was at least as effective when administered at one third of the dose. With the increased molecular mass of PEG-rHuMGDF, the relative potency of this form, on a molar basis, is even greater. However, the relative potencies of the two forms may vary in different settings.

Data presented in Fig 4 show by using both rHuG-CSF and PEGylated-rHuMGDF and delivering the combination by continuous infusion, it is possible to effect accelerated recovery of both neutrophils and platelets simultaneously. This represents a new possibility in combination cytokine treatment.

rHuG-CSF is capable of modulating platelet numbers as illustrated by recent clinical data: patients with untreated acute myeloid leukemia (AML) showed reduced platelet counts in response to rHuG-CSF administered at 10 μg/kg/d for 72 hours by continuous infusion.27 This phenomenon
would also appear to exist in mice since normal mice treated with rHuG-CSF at 200 μg/kg/d, again by continuous infusion, show decreased platelet counts.29 In the post-BMT setting studied here, rHuG-CSF again influenced circulating platelet numbers, manifest as a dampening of platelet recovery. This phenomenon was dose related. Figure 2 shows a dose of 72 μg/kg/d has a minimal impact on platelet recovery. Data shown in Fig 3 show that a dose three times greater results in a significant delay of platelet recovery. Combining PEG-rHuMGDF with either dose resulted in platelet recovery rates that differed only minimally from the enhanced response obtained with PEG-rHuMGDF alone (Fig 4). The magnitude of the enhancement in platelet recovery rate noted in recipients of only PEG-rHuMGDF was thus slightly reduced when rHuG-CSF was included in the posttransplant treatment. This phenomenon is not confined to rHuG-CSF; in fact, rmGM-CSF appears to supress platelet counts still further.29 This putative interlineage effect of either CSF is not confined to effects on platelets. Several reports have also documented an effect on erythropoiesis.30-33 Whether these phenomena represent an illustration of lineage competition or "steal" is unclear. The reduction noted in platelet counts in this work is not related to leukocyte counts since the degree of leukocytosis induced by 72 versus 200 μg/kg/d rHuG-CSF is of similar magnitude, yet only the higher dose reduced platelets. This argues against excessive "pull" down the neutrophil lineage, reducing input to the thrombocyte lineage. PEG-rHuMGDF does not have a negative impact on leucocyte or erythrocyte numbers despite causing a marked thrombocytosis. From a single report,24 it would appear that erythropoietin at relatively high treatment levels reduces circulating leucocyte numbers. High G-CSF treatment levels in turn reduced erythrocyte numbers (platelet numbers were not reported).24 This study used splenectomized mice, which may account for some of the effects. At this stage it is clear that when using "lineage restricted" growth factors such as G-CSF and erythropoietin, there is indeed an effect on lineages other than the main hematopoietic target population. Whether this phenomenon is reproducible in humans, and if it should be, whether it might be of sufficient magnitude to have therapeutic consequences remains to be seen. It is possible that differences between the physiology of mice and humans, discussed by Cronkite et al,35 may account for the observations. Also, the doses used in many mouse studies may be unrealistically high. However, none of these interlineage effects can be reproduced in vitro, implying an indirect effect. Since no similar reports have emerged from the widespread use of CSFs in the clinic, the validity of the mouse models may be called into question.

The most recent clinical practice has been to use peripheral blood progenitor cells (PBPC) as either an alternative source, or in support of BMT (eg, Sheridan et al35). This strategy improves the rate of platelet recovery in addition to leukocyte recovery and significantly reduces the need for hospital inpatient care and antibiotic support. We have recently shown that murine PBPC recipients benefit considerably from posttransplant treatment with PEG-rHuMGDF.28 The magnitude of the PEG-rHuMGDF effect in PBPC recipient mice is considerably greater than noted here in BMT mice. In most aspects of hematopoietic transplantation, the mouse model has proved to be generally predictive of the response later obtained in the clinic. However, a notable exception is that the murine recipient of PBPC does not, in our hands at least, show blood cell recovery rates above the rates obtained in BMT recipients. This is in contrast to the clinical experience of PBPC versus BMT, where the recovery advantage gained with PBPC grafting is readily exploited in patients. Whether this dissimilarity stems from innate differences between species or from differing ablation regimes or previous treatment history is as yet unclear.

The data presented here indicate that MGDF shows a distinct advantage when administered to BMT recipients. Accelerated recovery, persistent and extended leukocytosis and thrombocytosis, and the absence of any detectable side effects indicate the utility of the rHuG-CSF/PEGrHuMGDF combination for improving the recovery of both neutrophils and platelets simultaneously in BMT recipients.

ACKNOWLEDGMENT

We acknowledge the expert technical assistance of Brent Shannon and thank Dr G. Morstyn for support and comments on these studies.

REFERENCES


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